

Tobramycin-Treated *Pseudomonas aeruginosa* PA14 Enhances *Streptococcus constellatus* 7155 Biofilm Formation in a Cystic Fibrosis Model System

Katherine E. Price,^a Amanda A. Naimie,^a Edward F. Griffin,^a Charles Bay,^b George A. O'Toole^a

Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire, USA^a; The Dartmouth Institute of Public Health, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire, USA^b

ABSTRACT

Cystic fibrosis (CF) is a human genetic disorder which results in a lung environment that is highly conducive to chronic microbial infection. Over the past decade, deep-sequencing studies have demonstrated that the CF lung can harbor a highly diverse polymicrobial community. We expanded our existing *in vitro* model of *Pseudomonas aeruginosa* biofilm formation on CF-derived airway cells to include this broader set of CF airway colonizers to investigate their contributions to CF lung disease, particularly as they relate to the antibiotic response of the population. Using this system, we identified an interspecies interaction between *P. aeruginosa*, a bacterium associated with declining lung function and worsening disease, and *Streptococcus constellatus*, a bacterium correlated with the onset of pulmonary exacerbations in CF patients. The growth rate and cytotoxicity of *S. constellatus* 7155 and *P. aeruginosa* PA14 were unchanged when grown together as mixed biofilms in the absence of antibiotics. However, the addition of tobramycin, the frontline maintenance therapy antibiotic for individuals with CF, to a mixed biofilm of *S. constellatus* 7155 and *P. aeruginosa* PA14 resulted in enhanced *S. constellatus* biofilm formation. Through a candidate genetic approach, we showed that *P. aeruginosa* rhamnolipids were reduced upon tobramycin exposure, allowing for *S. constellatus* 7155 biofilm enhancement, and monorhamnolipids were sufficient to reduce *S. constellatus* 7155 biofilm viability in the absence of tobramycin. While the findings presented here are specific to a biofilm of *S. constellatus* 7155 and *P. aeruginosa* PA14, they highlight the potential of polymicrobial interactions to impact antibiotic tolerance in unanticipated ways.

IMPORTANCE

Deep-sequencing studies have demonstrated that the CF lung can harbor a diverse polymicrobial community. By recapitulating the polymicrobial communities observed in the CF lung and identifying mechanisms of interspecies interactions, we have the potential to select the best therapy for a given bacterial community and reveal potential opportunities for novel therapeutic interventions. Using an *in vitro* model of bacterial infection on CF airway cells, we tested how a particular polymicrobial community grows, damages human cells, and responds to antibiotics in single and mixed infections. We describe here the mechanism of an interspecies interaction between two pathogens in the CF lung, *P. aeruginosa* and *S. constellatus*, which is potentiated by a commonly prescribed antibiotic, tobramycin.

Cystic fibrosis is a human genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Mutations in the CFTR gene result in a wide array of deleterious effects throughout the body, including chronic life-long respiratory infections, the primary cause of morbidity and mortality in patients with cystic fibrosis. Historically, chronic lung infections have been attributed to relatively few organisms, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Burkholderia cepacia* complex (1–3). However, deep-sequencing studies have revealed that this list of microbes underestimates and oversimplifies the polymicrobial communities that reside in the airways of patients with cystic fibrosis (CF) (4–10). Dozens of bacterial genera have been identified in a single sputum sample, including organisms not generally identified by conventional culturing, such as *Streptococcus* spp., and anaerobes, including *Prevotella* (4–14).

Interspecies interactions within polymicrobial communities can be cooperative and competitive and can be influenced by the presence of antibiotics (15–17). Of particular interest in the CF lung is the potential for interspecies interactions between *P. aeruginosa* and *Streptococcus* species. *P. aeruginosa* is the most

abundant and prevalent organism identified by deep-sequencing and culture-based methods in sputum samples from adult CF patients (4–10, 18). Colonization by *P. aeruginosa* is associated with worsening lung function and disease progression (1–3) and thus has received the most attention in studies of microbial pathogenesis in the CF lung.

Interestingly, several recent deep-sequencing studies identified *Streptococcus* spp. as among the top three most abundant and

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Address correspondence to George A. O'Toole, georgeo@dartmouth.edu.

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prevalent organisms in CF sputum, and in a study from our group, *Streptococcus* spp. were the predominant organisms in the sputum of ~50% of the patients at our CF center (4, 6, 9). Despite the high abundance and prevalence of *Streptococcus* spp. in CF sputum, the role that *Streptococcus* spp. play in disease is unclear. In a cross-sectional study, infection with *Streptococcus* was reported to be associated with better health (6). However, Sibley et al. (11, 12) and Parkins et al. (19) reported that high numbers of *Streptococcus milleri* group organisms (*S. anginosus*, *S. constellatus*, and *S. intermedius*) in sputum samples are correlated with the onset of pulmonary exacerbation (11, 12, 19). The *S. milleri* group can be identified in the sputum samples from comparatively healthy (stable) CF patients (6); therefore, the simple presence or absence of a given microbe does not accurately predict the virulence of a community. Thus, identifying potential pathogenic and/or health-promoting bacterial interactions within the CF sputum community requires more information than survey studies provide.

Here, we sought to analyze how the composition of a bacterial community contributes to its physiology, pathogenesis, and antibiotic response in a mixed-culture model of cystic fibrosis. Given the studies showing that the *S. milleri* group is associated with the onset of pulmonary exacerbations, we hypothesized that a mixed community of *P. aeruginosa* and members of the *S. milleri* group would exhibit increased virulence or increased antibiotic tolerance compared to that with each bacterium alone. To this end, we expanded our previously described coculture system of *P. aeruginosa* biofilm formation on the surface of CF airway cells (20–22) to include the *S. milleri* group and other microbes identified by deep sequencing as being highly abundant and prevalent. In addition, we examined mixed communities of *S. milleri* group organisms and *P. aeruginosa* and their responses to antibiotic exposure. With this expanded system, we identified and characterized one example of interspecies interaction between *P. aeruginosa* PA14 and *S. constellatus* 7155 when treated with the commonly prescribed antibiotic tobramycin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *P. aeruginosa* strains used in this study are listed in Table S1 in the supplemental material and were grown on LB agar or liquid supplemented with 25 µg/ml gentamicin, 100 µg/ml carbenicillin, and where appropriate, shaking at 37°C. *S. constellatus* 7155 was grown on blood agar and Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) and 20 µl/ml Oxyrase (Oxyrase, Inc.) under static conditions at 37°C in a 5% CO₂ atmosphere. For mixed-culture assays, *P. aeruginosa* was selected on *Pseudomonas* isolation agar at 37°C, and *S. constellatus* was enriched on tryptic soy agar (TSA) supplemented with 5% defibrinated sheep's blood (blood agar) and grown anaerobically in GasPak Jars at 37°C. Information relevant to the growth of the other microbes mentioned in the manuscript is detailed in the supplemental material.

Tissue culture cultivation. The cystic fibrosis bronchial epithelial (CFBE) cell line used in this study overexpresses F508del-cystic fibrosis transmembrane conductance regulator (CFTR) via stable lentiviral transfection of human bronchial epithelial cells (23). The parent cell line, named CFBE41o⁺, was isolated from a CF patient who was homozygous for the F508del-CFTR mutation and was originally characterized by Bruscia and colleagues (24) and Cozens et al. (25). CFBE cells, here referred to as CF airway cells, were the generous gift of J. P. Clancy. CF airway cells were cultivated as previously described (20, 22). Briefly, CF airway cells were seeded into 24-well plates at 50,000 cells/well and fed every other day with minimal essential medium (MEM) (Life Technologies) supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin,

2 µg/ml puromycin, 5 µg/ml Plasmocin (InvivoGen), and 10% fetal bovine serum until confluent and tight junctions had formed (5 to 7 days).

Growth kinetics of biofilms formed on CF airway cells. Assays of biofilms formed on CF airway cells were performed as previously described (20–22), with the following modifications. An overnight culture of *P. aeruginosa* or *S. constellatus* was washed and resuspended in 1 ml of MEM. A *P. aeruginosa* inoculum was prepared to an optical density at 600 nm (OD₆₀₀) of 0.05 (~5 × 10⁷ CFU/ml), and an *S. constellatus* inoculum was prepared to an OD₆₀₀ of 0.1 (~5 × 10⁷ CFU/ml), both in MEM supplemented with 2 mM L-glutamine. A 1:1 mixture of *P. aeruginosa* and *S. constellatus* was prepared from these suspensions. Half a milliliter of each suspension (*P. aeruginosa* alone, *S. constellatus* alone, and a 1:1 mixture) was then gently added to each well of CF airway cells that had been washed twice with MEM. The cocultures were incubated 1 h at 37°C in 5% CO₂. At 1 h, unattached bacteria were removed by aspiration and the medium replaced with MEM supplemented with 2 mM L-glutamine and 0.4% arginine. Biofilms were allowed to incubate for a total of 1, 3, or 6 h. At each time point, planktonic cells were removed by aspiration, and the remaining biofilms were washed once with MEM supplemented with 2 mM L-glutamine and 0.4% arginine. Fresh MEM supplemented with 2 mM L-glutamine and 0.4% arginine was added to the wells before scraping biofilms with a pipette tip. Biofilm-grown bacteria were serially diluted and plated on *Pseudomonas* isolation agar (PIA) and blood agar, as described above, to identify *P. aeruginosa* and *Streptococcus* spp., respectively. PIA was incubated overnight at 37°C, and blood agar was incubated overnight anaerobically in GasPak jars at 37°C. After overnight incubation, the resulting colonies were counted and the CFU determined.

Biofilm antibiotic assay. Coculture biofilm assays were performed as previously described (20), with the following modifications. The coculture assays were inoculated as described above in the growth kinetic assays. One hour postinoculation, unattached cells were removed by aspiration and the medium replaced with MEM supplemented with 2 mM L-glutamine and 0.4% arginine. Six hours postinoculation, the unattached bacteria were removed by aspiration; the biofilm fraction was washed once with MEM supplemented with 2 mM L-glutamine and 0.4% arginine and then incubated with or without 5 µg/ml tobramycin in MEM supplemented with 2 mM L-glutamine and 0.4% arginine. Twenty-one hours postinoculation, planktonic fractions were removed and saved for use in a drop collapse assay. MEM supplemented with 2 mM L-glutamine and 0.4% arginine was added to the wells, and biofilms were disrupted by scraping with a pipette tip. The biofilm-grown bacteria were serially diluted and then plated on PIA and blood agar to distinguish the microbes, as described above. PIA was incubated overnight at 37°C, and blood agar was incubated anaerobically overnight at 37°C in GasPak jars. After overnight incubation, the resulting colonies were counted and the CFU determined. Antibiotic assays on plastic were performed exactly as on CF airway cells, except biofilms were formed directly on plastic tissue culture-treated 24-well plates. When indicated, rhamnolipids (50 µg/ml, dissolved in MEM; Sigma) were added to washed preformed biofilms at 6 h postinoculation. When indicated, heat-killed wild-type *P. aeruginosa* cells were added to the washed preformed biofilms at 6 h postinoculation. Heat-killed *P. aeruginosa* was prepared from an overnight culture grown in LB that was washed once and resuspended in 1 ml of MEM. The OD₆₀₀ was calculated for this suspension, and an appropriate volume of cells was heat killed at 80°C for 10 min. The heat-killed bacteria were then resuspended in MEM supplemented with 2 mM L-glutamine and 0.4% arginine with or without tobramycin and added to the washed preformed biofilms.

Microscopy. MatTek dishes were seeded with 200,000 CF airway cells/dish and grown until tight junctions formed (5 to 7 days), as described previously (20–22). *P. aeruginosa* PA14 harboring a constitutively expressed *gfp* gene (26) and *S. constellatus* 7155 were prepared as described for the coculture assays and added to twice-washed CF airway cells. Unattached bacteria were removed after 1 h, and the medium was replaced with MEM supplemented with 2 mM L-glutamine and 0.4% arginine. At 3 and 4 h postinoculation, the unattached bacteria were removed, and the

medium was replaced with MEM supplemented with 2 mM L-glutamine, 0.4% arginine, and 2.5 μ l of a 3.125% hexidium iodine solution (which selectively stains Gram-positive bacteria red to stain *S. constellatus*) and incubated for 15 min at 37°C in 5% CO₂. Single-strain and mixed biofilms were visualized on a Nikon Eclipse Ti inverted microscope with a 100 \times objective, and images were acquired using the Nikon ND acquisition software and a Hamamatsu C11440 Orca-Flash 4.0 digital camera. *P. aeruginosa* was visualized via its green fluorescence with the green fluorescent protein (GFP) channel of the ND acquisition software; *S. constellatus* was visualized via its red fluorescence in the mCherry channel of the ND acquisition software.

Drop collapse assay. Drop collapse assays were performed as previously described (27, 28). Briefly, planktonic fractions from biofilm assays were collected and clarified by centrifugation. The clarified supernatants were serially diluted (1:1) with water in a 96-well plate. Thirty microliters of each dilution was spotted onto the circles on the underside of the lid of the 96-well plate and assessed for surfactant activity, as measured by the spread of the droplet. As surfactant quantities are reduced by dilution, surface tension increases, resulting in the beading up of the droplet. Surfactant scores are equal to the reciprocal of the greatest dilution at which there was surfactant activity (a collapsed drop). A score of 1 is assigned to surfactant activity in the undiluted supernatant of the wild-type strain.

Cytotoxicity assays. Cytotoxicity was measured as fraction of lactate dehydrogenase (LDH) release compared to total lysis control of uninfected CF airway cells treated with Triton X-100 detergent to completely lyse cells and release all intracellular LDH using the Cyto Tox 96 nonradioactive cytotoxicity kit (Promega), according to the manufacturer's instructions.

RESULTS

Expanded model system of bacterial biofilm formation on CF airway cells. Our existing model system of bacterial biofilm formation on the surface of CF-derived bronchial epithelial (CFBE) cells was originally designed to study *P. aeruginosa* biofilms. *P. aeruginosa* grown in this model system recapitulates several key aspects of chronic biofilm formation, including the formation of biofilm-like microcolonies, expression of genes associated with biofilm growth, induction of quorum sensing, requirement for genes necessary for biofilm formation on abiotic surfaces, and, of importance in the clinic, increased antibiotic tolerance (20, 22). We expanded this system to assess the biofilm-forming ability of seven *Streptococcus* species and other microbes identified by deep-sequencing studies as highly abundant and/or prevalent in sputum from CF patients, including *Prevotella intermedia*, *S. aureus*, *Fusobacterium nucleatum*, *Stenotrophomonas maltophilia*, and *Gemella* species (4–6, 9). Each species was inoculated individually onto CF airway cells for a total of 1, 3, or 6 h and assessed for bacterial viability. Fifteen of the 16 species tested were able to grow on the surface of CF airway cells (see Fig. S1A in the supplemental material). We were unable to recover viable bacteria from obligate anaerobe *F. nucleatum* cultures on CF airway cells, likely because our culturing conditions were not anoxic (see Fig. S1A).

Given that *P. aeruginosa* and *S. milleri* group organisms may influence the overall pathogenesis in the CF lung (1–3, 6, 11, 12, 14, 19), we sought to assess whether *P. aeruginosa* and *Streptococcus* interactions might impact cell growth or cytotoxicity to host cells. Pairwise combinations of *P. aeruginosa* and *S. milleri* group organisms were grown as biofilms on CF airway cells, assayed for their cytotoxicity and bacterial growth kinetics, and compared with respect to their single-species cytotoxicity and growth kinetics. *S. milleri* group and *P. aeruginosa* mixed biofilms on CF airway cells showed a level of cytotoxicity after 1 and 3 h of biofilm for-

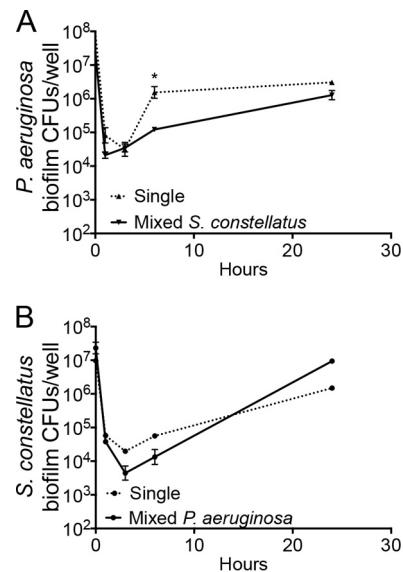


FIG 1 Growth kinetics of *P. aeruginosa* and *S. constellatus* in CF airway cells. (A) *P. aeruginosa* growth kinetics are largely unaffected by the presence of *S. constellatus* 7155. The dashed line indicates the viability of *P. aeruginosa* grown as single biofilms on CF airway cells, and the solid line indicates the viability of *P. aeruginosa* grown as mixed biofilms on CF airway cells with *S. constellatus* 7155. The only significant difference was noted at 6 h, with no significant difference at 24 h. *, $P < 0.05$. (B) *S. constellatus* 7155 growth kinetics are unaffected by the presence of *P. aeruginosa* PA14. The dashed line indicates viability of *S. constellatus* 7155 grown as single biofilms on CF airway cells, and the solid line indicates viability of *S. constellatus* 7155 grown as mixed biofilms on CF airway cells with *P. aeruginosa*. For all panels, the symbols and error bars indicate the means and standard deviations (SD) of the results from three biological replicates.

mation similar to that seen with single *P. aeruginosa* biofilms. At 6 h, the two strains of *S. constellatus* showed similar levels of cytotoxicity toward host cells, while *S. anginosus* and *S. intermedius* showed reduced levels of cytotoxicity toward host cells compared to those with single *P. aeruginosa* biofilms formed on CF airway cells (see Fig. S1B in the supplemental material). *P. aeruginosa* growth was largely unaffected by growth in the presence of the four streptococcal strains tested (Fig. 1A; see also Fig. S1C in the supplemental material). We did observe a modest but significant reduction of *P. aeruginosa* growth at 6 h (t test, $P < 0.05$) when this microbe was cocultured with *S. constellatus* 7155 (Fig. 1A), but no difference in CFU was observed by 24 h. While coculture with *P. aeruginosa* did not impact the growth of *S. constellatus* 7155 (Fig. 1B), *S. constellatus* C818, or *S. anginosus*, we did notice a significant impact of growth on a coculture of *S. intermedius* with *P. aeruginosa*, as described in Fig. S1D to F in the supplemental material.

Tobramycin treatment enhances growth of *S. constellatus* 7155 in a mixed biofilm with *P. aeruginosa*. Individuals with CF receive aerosolized antibiotics on a regular basis as part of a maintenance therapy regimen and intravenous antibiotics upon admission to the hospital during a pulmonary exacerbation. We therefore hypothesized that antibiotic treatment would affect the physiology and/or pathogenesis of *P. aeruginosa* or the *S. milleri* group in mixed biofilms. To test this idea, we established single and mixed biofilms of *P. aeruginosa* and *S. constellatus*, *S. anginosus*, or *S. intermedius* for 6 h on CF airway cells before treating the

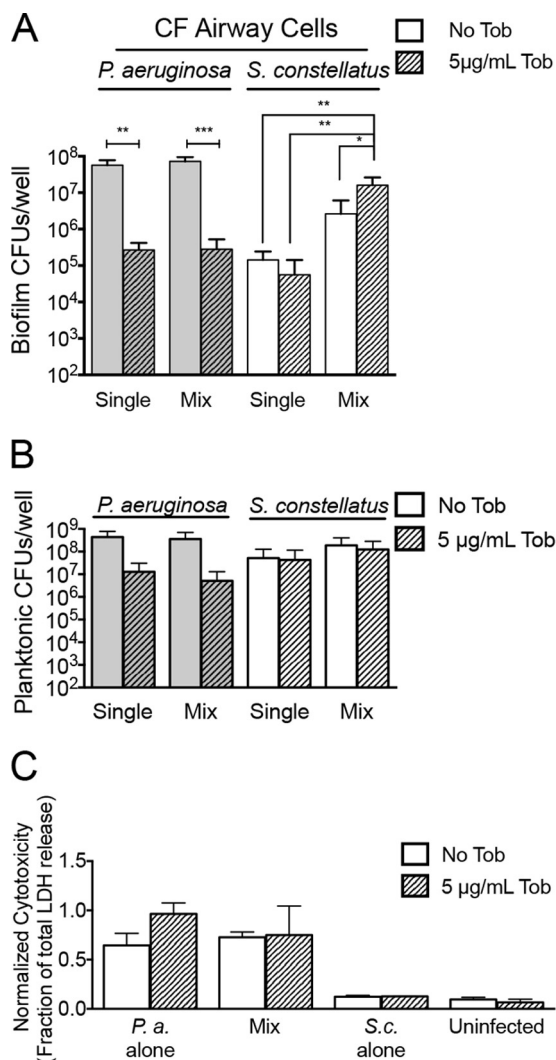


FIG 2 Tobramycin (Tob) treatment of *P. aeruginosa* and *S. constellatus* mixed biofilms enhances *S. constellatus* viability. The data represent the viability of bacteria grown as biofilms at 21 h on CF airway cells from assays of single or mixed biofilms of *P. aeruginosa* and *S. constellatus* 7155 treated with no tobramycin or with 5 µg/ml tobramycin. (A) Viability of bacteria in the biofilm fraction. (B) Viability of bacteria in the planktonic fraction. (C) Cytotoxicity of indicated mixed biofilms on CF airway cells. Cytotoxicity was normalized to total cell lysis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, as determined by analysis of variance (ANOVA) with Tukey's posttest for multiple comparisons. *P.a.*, *P. aeruginosa*; *S.c.*, *S. constellatus*. For all panels, the bars and error bars indicate the means and SD of the results from three biological replicates.

established biofilms with 5 µg/ml tobramycin or growth medium as a control, as previously reported (20–22). After 15 h of antibiotic treatment, bacterial biofilm viability (measured as the number of CFU per well) was determined for the mixed community and compared to the bacterial biofilm viability for each organism grown individually under the same conditions.

Viable *P. aeruginosa* levels decreased >200-fold when *P. aeruginosa* was treated with 5 µg/ml tobramycin and grown alone or in mixed biofilms with *S. constellatus* 7155 (Fig. 2A). *S. constellatus* 7155 grown alone on CF airway cells showed a 2.5-fold decline in viability when treated with 5 µg/ml tobramycin. A mixed biofilm of *S. constellatus* 7155 and *P. aeruginosa* in the absence of

tobramycin resulted in a nonsignificant trend toward enhanced *S. constellatus* 7155 viability similar to that seen with single *S. constellatus* 7155 biofilms after 21 h of growth. However, when mixed biofilms of *S. constellatus* 7155 and *P. aeruginosa* were treated with tobramycin, the viability of *S. constellatus* 7155 statistically significantly increased 6-fold compared to that of the mixed community without tobramycin and 292-fold compared to that of single *S. constellatus* 7155 biofilms in the presence of this antibiotic (Fig. 2A). Thus, the presence of *P. aeruginosa* PA14 may be beneficial for *S. constellatus* 7155 when this microbe is grown in biofilms on CF airway cells, and *S. constellatus* 7155 clearly benefits when the community is treated with tobramycin. Interestingly, we saw no differences in single and mixed cultures with or without tobramycin when we examined the planktonic population (Fig. 2B) or cytotoxicity (Fig. 2C).

The coculture of *S. constellatus* 7155 with *P. aeruginosa* PAO1 did not show a significant enhancement in *S. constellatus* 7155 growth in the presence or absence of tobramycin (see Fig. S2A in the supplemental material). We also found no evidence of a tobramycin-dependent interaction with *P. aeruginosa* PA14 when tested with two other members of the *S. milleri* group, *S. intermedius* and *S. anginosus*, or with *S. constellatus* C818 (see Fig. S2B in the supplemental material), indicating that the observed phenotype was specific for this strain of *S. constellatus* (see Discussion).

Characterization of tobramycin-dependent enhancement of *S. constellatus* 7155 in mixed biofilms with *P. aeruginosa* PA14. One explanation for the increase in *S. constellatus* 7155 CFU in mixed biofilm with *P. aeruginosa* treated with tobramycin may be enhanced nutrient availability. That is, tobramycin reduced viable *P. aeruginosa* levels in this assay by 256-fold; it is possible that these dead *P. aeruginosa* serve as a source of nutrients for *S. constellatus* 7155, thus allowing for their increased viability. To address this possibility, heat-killed *P. aeruginosa* was added to single *S. constellatus* 7155 biofilms grown on CF airway cells at the same time as the addition of antibiotic to mimic the availability of nutrients from dying *P. aeruginosa*. *S. constellatus* 7155 biofilms that received heat-killed *P. aeruginosa* had significantly lower viable counts than those of *S. constellatus* 7155 grown as mixed biofilms with live *P. aeruginosa* (Fig. 3A). Additionally, the viability of *S. constellatus* 7155 that received heat-killed *P. aeruginosa* did not increase upon tobramycin treatment (Fig. 3A).

We next examined *P. aeruginosa* PA14 and *S. constellatus* 7155 growing as biofilms on CF airway cells via epifluorescence microscopy. We reasoned that understanding the physical proximity of *S. constellatus* 7155 and *P. aeruginosa* in mixed biofilms on CF airway cells would be informative in understanding the mechanism of this interspecies interaction. In these studies, *P. aeruginosa* PA14 was visualized by its green fluorescence from a GFP constitutively expressed from a plasmid (26), while *S. constellatus* 7155 was visualized by its red fluorescence after staining with hexidium iodide, which preferentially stains Gram-positive bacteria (Fig. 3C and D) (29). When examined by phase contrast and fluorescence microscopy, *P. aeruginosa* and *S. constellatus* 7155 were found to colocalize on the surface of CF airway cells *in vitro* (Fig. 3B). Colocalization was not required for biofilm formation, as single-species biofilms of *P. aeruginosa* or *S. constellatus* 7155 were also visualized in mixed biofilms (Fig. 3B) and single-species biofilms (Fig. 3C and D).

To determine if the impact of tobramycin on *S. constellatus* 7155 when grown in mixed biofilms with *P. aeruginosa* on CF

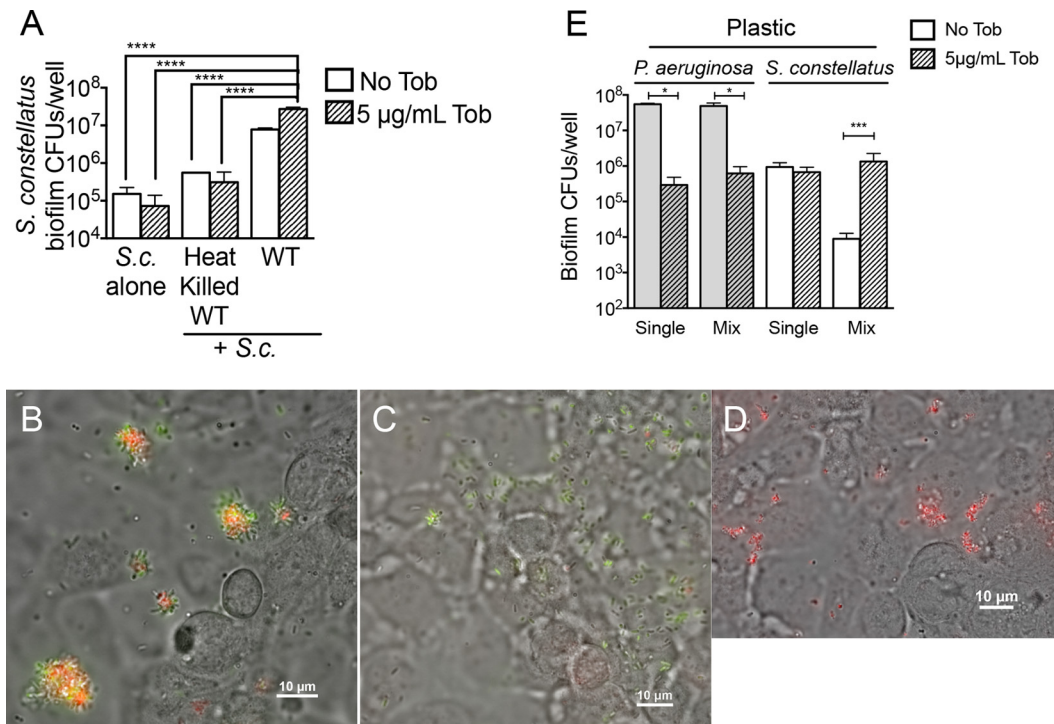


FIG 3 Characterization of tobramycin enhancement of *S. constellatus* in mixed biofilms with *P. aeruginosa*. (A) Viability of *S. constellatus* 7155 grown as biofilms on CF airway cells from assays of single or mixed biofilms of heat-killed *P. aeruginosa* PA14 or non-heat-killed *P. aeruginosa* PA14, as indicated, treated with no tobramycin or with 5 µg/ml tobramycin. ****, $P < 0.0001$, as determined by ANOVA, with Tukey's posttest for multiple comparisons. WT, wild type. (B) *P. aeruginosa* and *S. constellatus* colocalize *in vitro*. Shown is a representative image of a mixed-species biofilm of *P. aeruginosa* (green, expressing GFP) and *S. constellatus* 7155 (red, stained with hexidium iodide) on the surface of CF airway cells (gray) at ~3 h postinoculation with *P. aeruginosa* and *S. constellatus* 7155. (C) Representative image of a single-species biofilm of *P. aeruginosa* (green, expressing GFP) on the surface of CF airway cells (gray) at ~3 h postinoculation with *P. aeruginosa* alone. Red staining indicates the nonspecific staining levels of hexidium iodide for CF airway cells. (D) Representative image of *S. constellatus* 7155 (red, stained with hexidium iodide) on the surface of CF airway cells (gray) at ~3 h postinoculation with *S. constellatus* 7155 alone. (E) Tobramycin treatment of *P. aeruginosa* and *S. constellatus* 7155 mixed biofilms enhances *S. constellatus* biofilm formation on plastic. The data represent the viability of bacteria grown as biofilms on plastic from biofilm assays on single or mixed biofilms of *P. aeruginosa* and *S. constellatus* 7155 treated with no tobramycin or 5 µg/ml tobramycin. *, $P < 0.05$; ***, $P < 0.001$, as determined by the Mann-Whitney test. For panels A and E, the bars and error bars indicate the means and SD of the results from three biological replicates.

airway cells was dependent upon the presence of host cells, the biofilm assays described above were repeated for biofilms grown on the plastic surface of 24-well plates rather than a monolayer of CF airway cells. Levels of *P. aeruginosa* grown alone or in mixed biofilms on plastic decreased by 187-fold and 78-fold with tobramycin treatment (Fig. 3E), respectively, recapitulating the findings obtained from CF airway cell-grown biofilms (Fig. 2). Similarly, the growth and antibiotic response of plastic-grown *S. constellatus* 7155 single-species biofilms were comparable to those observed for CF airway cell-grown *S. constellatus* 7155 single-species biofilms, decreasing by 1.4-fold (Fig. 3E). In contrast, mixed biofilms of *S. constellatus* 7155 and *P. aeruginosa* on plastic resulted in a sharp 105-fold decline in the numbers of *S. constellatus* 7155 without tobramycin treatment compared to untreated single *S. constellatus* 7155 biofilms. Interestingly, a 150-fold increase in *S. constellatus* 7155 viability was observed in the presence of *P. aeruginosa* during tobramycin treatment of these mixed-species plastic-grown biofilms (Fig. 3E), a finding similar to that shown for CF airway cell-grown biofilms (Fig. 2A).

In summary, we demonstrated that heat-killed *P. aeruginosa* cannot recapitulate the tobramycin-dependent enhancement of *S. constellatus* 7155 growth in mixed biofilms with *P. aeruginosa* and that *S. constellatus* 7155 colocalizes with *P. aeruginosa* on CF air-

way cells. Studies of biofilms grown on plastic indicate that *P. aeruginosa* inhibited the growth of *S. constellatus* 7155 on plastic, but this inhibition could be relieved by the addition of tobramycin. The growth of biofilms on CF airway cells is somewhat more complex. In the absence of tobramycin, *S. constellatus* 7155 shows a stimulation of growth in the presence of *P. aeruginosa* which is further enhanced upon treatment with tobramycin, indicating a potential multifactorial interaction between these two microbes when grown on CF airway cells. In the subsequent studies described below, we focus on the ability of tobramycin to enhance the population of *S. constellatus* 7155 when this microbe is grown in the presence of *P. aeruginosa* PA14, thereby allowing us to perform our assays on plastic or on CFBE cells.

Screening for *P. aeruginosa* genes responsible for tobramycin-dependent *S. constellatus* biofilm growth enhancement using a candidate gene approach. We hypothesized that the increase in *S. constellatus* 7155 biofilm viability when treated with tobramycin in mixed biofilms with *P. aeruginosa* PA14 was due to a tobramycin-dependent change in gene expression by *P. aeruginosa*, consistent with several studies demonstrating that *P. aeruginosa* gene expression changes in response to tobramycin treatment (20, 30–36). Specifically, we hypothesized that tobramycin treatment activates one or more *P. aeruginosa* genes that enhance

S. constellatus 7155 biofilm viability, or alternatively, that tobramycin treatment could repress one or more *P. aeruginosa* genes that diminish *S. constellatus* 7155 biofilm viability. From a list of candidate genes chosen for their reported change in expression in response to tobramycin treatment, role in biofilm formation, and/or antibiotic tolerance (see Table S2 in the supplemental material), we performed a screen with mutant *P. aeruginosa* PA14 strains and wild-type *S. constellatus* 7155 to identify *P. aeruginosa* genes responsible for enhancing *S. constellatus* 7155 survival in mixed biofilms on plastic when treated with tobramycin. We performed the first round of this screen on plastic because growth on plastic served as a facile experimental tool, as this assay allowed us to focus on the *P. aeruginosa*-mediated growth inhibition of *S. constellatus* 7155 and the relief of that inhibition upon treatment with tobramycin. Forty-three mutant *P. aeruginosa* strains were tested in the screen with wild-type *S. constellatus* 7155 grown as biofilms on plastic (see Table S2); a mixture of the wild-type *P. aeruginosa* with wild-type *S. constellatus* 7155 served as the control.

Several genes related to rhamnolipid production were identified as candidates for mutations capable of enhancing *S. constellatus* 7155 growth in the absence of tobramycin treatment; that is, the viable counts of *S. constellatus* 7155 were equally high with or without tobramycin when cultured with *P. aeruginosa* strains carrying these mutations on plastic. Rhamnolipids are produced by the *rhIA*, *rhIB*, and *rhIC* gene products, which are responsible for the biosynthesis of β -hydroxyalkanoyl- β -hydroxyalkanoic acids (HAAs), monorhamnolipids, and dirhamnolipids, respectively, all of which are surfactants secreted by *P. aeruginosa* (Fig. 4A). The *rhIA* and *rhIB* genes are organized in an operon and transcriptionally induced by the activated quorum-sensing regulator RhlR. The *rhIC* gene is located elsewhere on the chromosome and is also activated by RhlR (27, 37–40).

Mixed biofilms of *S. constellatus* 7155 with *P. aeruginosa* PA14 strains carrying mutations in the *rhIA* or *rhIB* genes resulted in high *S. constellatus* 7155 viability without the addition of tobramycin on plastic (Fig. 4B), a phenotype suggesting that tobramycin repressed the production of HAAs and monorhamnolipids. Consistent with this finding, mixed biofilms of *S. constellatus* 7155 and *P. aeruginosa* PA14 mutants with defects in the transcriptional activators of *rhIA* and *rhIB* (the *lasR*, *rhlR*, and *lasR* *rhlR* mutants) also resulted in high *S. constellatus* 7155 biofilm viability without the addition of tobramycin (see Table S2 in the supplemental material). Mixed biofilms of *S. constellatus* 7155 and a *P. aeruginosa* PA14 strain carrying a mutation in the *rhIC* gene did not result in an increase in the level of *S. constellatus* 7155 without the addition of tobramycin (Fig. 4B), phenocopying mixed biofilms of *S. constellatus* 7155 and wild-type *P. aeruginosa* PA14. This result suggested that HAAs and/or monorhamnolipids produced by the *rhIC* *P. aeruginosa* strain were sufficient to repress *S. constellatus* 7155.

To address whether tobramycin did indeed reduce the production of surfactants by *P. aeruginosa*, bulk surfactant production was measured by a drop collapse assay, as previously described (27, 28), to measure the effect of tobramycin on surfactant secretion into the supernatant. In a representative assay, mixed biofilms of *S. constellatus* 7155 and wild-type *P. aeruginosa* PA14 resulted in a surfactant score of 1.0 without tobramycin treatment and results below the limit of detection with tobramycin treatment (Fig. 4C). Mixed biofilms of *S. constellatus* 7155 and the *P.*

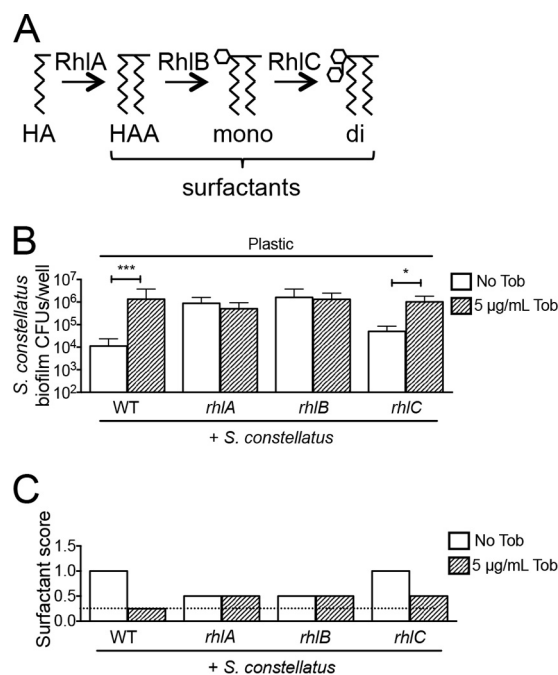


FIG 4 Monorhamnolipids prevent *S. constellatus* biofilm formation in mixed biofilms with *P. aeruginosa* on plastic. (A) Diagram of rhamnolipid biosynthetic pathway and the proteins required for each step in the biochemical pathway. Mutating *rhIA* would result in an accumulation of hydroxyacyl-ACP (HA), mutating *rhIB* results in the accumulation of β -hydroxyalkanoyl- β -hydroxyalkanoic acid (HAA), and the loss of *rhIC* causes the accumulation of monorhamnolipids (mono). di, dirhamnolipid. (B) Viability of *S. constellatus* 7155 grown as mixed biofilms on plastic from biofilm assays of *S. constellatus* 7155 with the wild type or the *rhIA*, *rhIB*, or *rhIC* mutant of *P. aeruginosa*, not treated with tobramycin or treated with 5 μ g/ml tobramycin. The bars and error bars indicate the means and SD of the results from three biological replicates. ***, $P < 0.001$; *, $P < 0.05$, as determined by the Mann-Whitney test. (C) Representative surfactant score from assays of mixed biofilms of *S. constellatus* 7155 with the wild type or the *rhIA*, *rhIB*, or *rhIC* mutant of *P. aeruginosa* on plastic, not treated with tobramycin or treated with 5 μ g/ml tobramycin. The dashed line indicates the limit of detection.

aeruginosa PA14 *rhIA* mutant or the *rhIB* mutant resulted in a surfactant score of 0.5 with and without tobramycin treatment (Fig. 4C). A mixed biofilm of *S. constellatus* 7155 and the *P. aeruginosa* PA14 *rhIC* mutant showed surfactant scores of 1.0 without tobramycin treatment and 0.5 with tobramycin treatment (Fig. 4C). These results suggested that tobramycin interferes with rhamnolipid production and that the surfactants produced by the *P. aeruginosa* PA14 *rhIC* mutant are sufficient to inhibit *S. constellatus* 7155 biofilms. Thus, we propose that rhamnolipids negatively impact *S. constellatus* 7155 viability in a biofilm formed on plastic, monorhamnolipids are sufficient for this effect, and the loss of rhamnolipid production by *P. aeruginosa* PA14 upon tobramycin treatment allows for the increased *S. constellatus* 7155 observed in our study.

***P. aeruginosa*-produced monorhamnolipids inhibit *S. constellatus* biofilms on airway cells.** We next asked if rhamnolipids reduced *S. constellatus* 7155 CFU in mixed biofilms with *P. aeruginosa* PA14 formed on the more clinically relevant CF airway cells. Mixed biofilms of *S. constellatus* 7155 and the *P. aeruginosa* PA14 strains with mutations in the *rhIA* or *rhIB* genes formed on airway cells resulted in a level of *S. constellatus* 7155 viability without

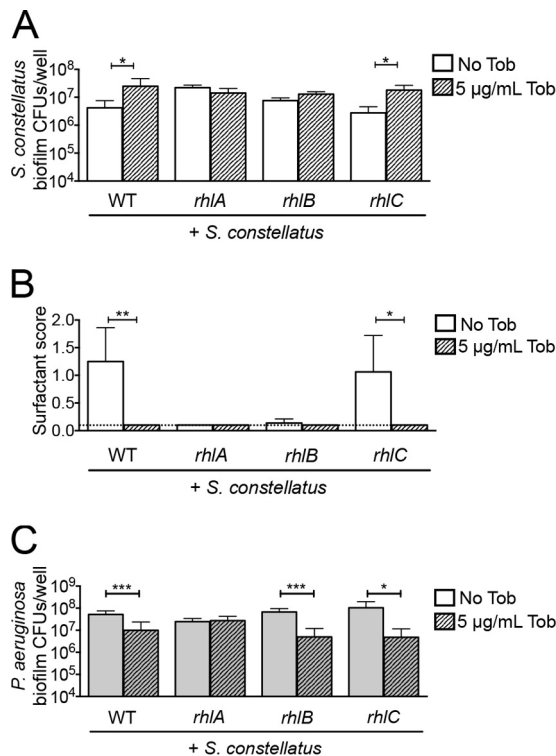


FIG 5 Monorhamnolipids reduce *S. constellatus* biofilm viability in mixed culture with *P. aeruginosa* on CF airway cells. (A) Viability of *S. constellatus* 7155 grown as mixed biofilms on CF airway cells with the wild type or the *rhIA*, *rhIB*, or *rhIC* mutant of *P. aeruginosa* not treated with tobramycin or treated with 5 µg/ml tobramycin. (B) Surfactant score from assays of mixed biofilms of *S. constellatus* 7155 with the wild type or the *rhIA*, *rhIB*, or *rhIC* mutant of *P. aeruginosa* on CF airway cells not treated with tobramycin or treated with 5 µg/ml tobramycin. The dashed line indicates the limit of detection. (C) Viability of the wild type or the *rhIA*, *rhIB*, or *rhIC* mutant of *P. aeruginosa* grown as mixed biofilms on CF airway cells with *S. constellatus* 7155 not treated with tobramycin or treated with 5 µg/ml tobramycin. For all panels, the bars and error bars indicate the means and SD of the results from three biological replicates. *, $P < 0.05$; **, $P < 0.01$, as determined by an unpaired *t* test.

tobramycin (Fig. 5A) which is typically observed for this organism only when grown in the presence of *P. aeruginosa* PA14 with tobramycin. Mixed biofilms of *S. constellatus* 7155 and the *P. aeruginosa* PA14 *rhIC* mutant formed on airway cells did not result in increased *S. constellatus* 7155 CFU without tobramycin (Fig. 5A). Similar to what we observed on plastic, when grown as mixed biofilms on airway cells, *S. constellatus* 7155 CFU were the highest in mixed biofilms in which surfactant levels were the lowest (Fig. 5A and B). These results suggest that the surfactants produced by the *P. aeruginosa* *rhIC* mutant are sufficient to repress *S. constellatus* 7155 growth on airway cells.

It is possible that the tobramycin-dependent increase in *S. constellatus* 7155 viability in mixed biofilms formed on airway cells was due to an increased opportunity to colonize and/or additional nutrients that were available due to the reduced population of *P. aeruginosa* in the tobramycin-treated mixed biofilm. Several lines of evidence argue against this idea. First, single *S. constellatus* 7155 biofilms grown on CF airway cells, which should have the most available colonization surface and nutrients, have smaller amounts of viable *S. constellatus* 7155 than mixed biofilms of *S. constellatus* 7155 and *P. aeruginosa* (Fig. 2A). Second, adding heat-

killed *P. aeruginosa* to an *S. constellatus* 7155 biofilm grown on CF airway cells did not result in increased *S. constellatus* 7155 viability (Fig. 3A). Third, the *rhIA* mutant used in these studies harbors a gentamicin resistance cassette in place of the *rhIA* gene, conferring cross-resistance to tobramycin to this mutant. In mixed biofilms of *S. constellatus* 7155 and the *P. aeruginosa* *rhIA* mutant, there are high levels of *S. constellatus* 7155 and *P. aeruginosa* with and without the addition of tobramycin (Fig. 5A and C). These results suggest that the growth enhancement of *S. constellatus* 7155 is not promoted by the absence of *P. aeruginosa*. Instead, these genetic studies support the model that *P. aeruginosa*-secreted rhamnolipids inhibit *S. constellatus* 7155 biofilm formed on CF airway cells and that surfactant production can be repressed by the addition of tobramycin.

Exogenous rhamnolipids inhibit *S. constellatus* biofilm formation on airway cells. We further confirmed the role of rhamnolipids in reducing *S. constellatus* viability by adding purified commercially available rhamnolipids (a mixture of mono- and dirhamnolipids) to a mixed biofilm of *S. constellatus* 7155 and the *P. aeruginosa* *rhIA* mutant. Rhamnolipids were added at 50 µg/ml, a concentration that has been measured in broth-grown *P. aeruginosa* (41). The addition of exogenous rhamnolipids to the mixed biofilm of *S. constellatus* 7155 and the *P. aeruginosa* *rhIA* mutant represses *S. constellatus* 7155 CFU to the same extent as wild-type *P. aeruginosa* without tobramycin treatment, effectively complementing the *rhIA* mutant phenotype (Fig. 6A). Drop collapse assays on the supernatants from these biofilms revealed that 50 µg/ml exogenous rhamnolipids is comparable to the amount of surfactant produced by mixed biofilms of *S. constellatus* 7155 and *P. aeruginosa* (Fig. 6B). The addition of exogenous rhamnolipids did not impact *P. aeruginosa* viability (Fig. 6C) or cytotoxicity toward host cells (Fig. 6D). Taken together, our studies are consistent with the model that rhamnolipids produced by *P. aeruginosa*, or added exogenously, can reduce *S. constellatus* 7155 viability.

Rhamnolipids likely inhibit *S. constellatus* 7155 in mixed biofilms by direct killing. Rhamnolipids can disrupt biofilms by directly killing bacteria (42) and/or stimulating dispersion (41, 43). To test the hypothesis that rhamnolipids were dispersing *S. constellatus* 7155 biofilms, we measured the amount of *S. constellatus* 7155 in the planktonic fraction of the mixed biofilms formed on airway cells. In mixed biofilms of *S. constellatus* 7155 and wild-type *P. aeruginosa* or the *P. aeruginosa* *rhIA* mutant, we found no evidence of increased dispersion in mixed biofilms that produced rhamnolipids (*S. constellatus* 7155 and wild-type *P. aeruginosa*) compared to those that did not (*S. constellatus* 7155 and the *P. aeruginosa* *rhIA* mutant), as measured by viable *S. constellatus* 7155 levels in the planktonic fraction of CF airway cell-grown biofilms (Fig. 7A). Interestingly, we saw no statistically significant impact of the *rhIA* mutant on the viability of planktonic *S. constellatus* 7155, consistent with the data in Fig. 2B, indicating that the rhamnolipid-mediated reduction in the viability of *S. constellatus* 7155 by *P. aeruginosa* PA14 may be a biofilm-specific effect.

To test whether *S. constellatus* 7155 grown in a biofilm on CF airway cells was susceptible to direct killing by rhamnolipids, 50 µg/ml rhamnolipids was added to preformed single *S. constellatus* 7155 biofilms grown on CF airway cells. Adding rhamnolipids to single *S. constellatus* 7155 biofilms significantly reduced the viability of *S. constellatus* 7155 by 27-fold after 15 h of treatment of

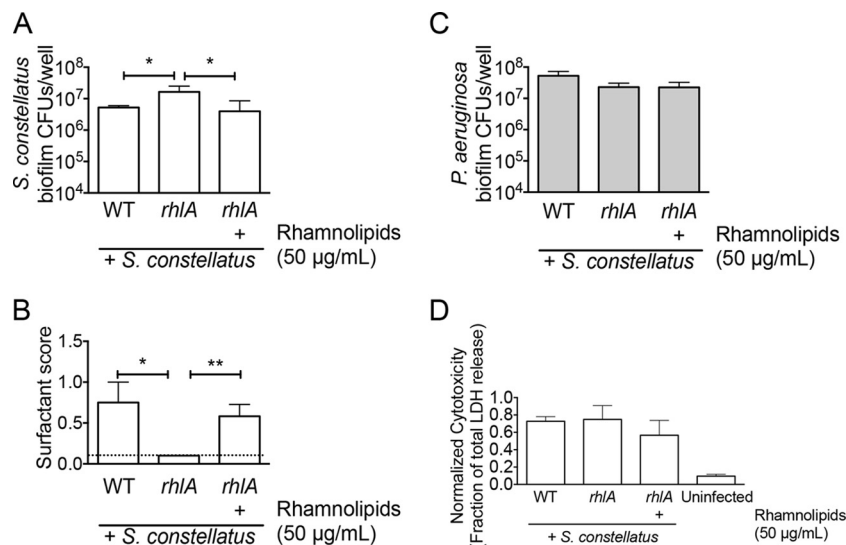


FIG 6 Exogenous rhamnolipids reduce *S. constellatus* viability in biofilms on CF airway cells. (A and B) Shown are the viability (A) and surfactant scores (B) of *S. constellatus* 7155 grown as single and mixed biofilms on CF airway cells with the wild type or the *rhIA* mutant of *P. aeruginosa* treated with 50 µg/ml rhamnolipids, as indicated. The dashed line indicates the limit of detection. (C) Viability of the wild type or the *rhIA* mutant *P. aeruginosa* grown as mixed biofilms on CF airway cells with *S. constellatus* 7155 treated with 50 µg/ml rhamnolipids, as indicated. (D) Cytotoxicity of indicated mixed biofilms on CF airway cells. Cytotoxicity was normalized to total cell lysis. For all panels, the bars and error bars indicate the means and SD of the results from three biological replicates. *, $P < 0.05$; **, $P < 0.01$, as determined by an unpaired *t* test.

biofilm-grown bacteria (Fig. 7B). Addition of rhamnolipids to the initial inoculum of a planktonically grown *S. constellatus* 7155 culture in THY resulted in an approximately 83,000-fold reduction in recovered CFU after 15 h of incubation (Fig. 7C). The kinetics of rhamnolipid-mediated killing is relatively rapid, with a decrease in the viability of *S. constellatus* 7155 planktonically

grown cells noted as early as 3 h after the addition of surfactant and an 8,200-fold reduction by 6 h postaddition (Fig. 7D). Thus, the concentration of rhamnolipids produced by *P. aeruginosa* in broth culture (41) and in a mixed biofilm with *S. constellatus* 7155 on CF airway cells (Fig. 6B) is sufficient to significantly reduce the viability of *S. constellatus* 7155 grown as a biofilm on CF airway cells.

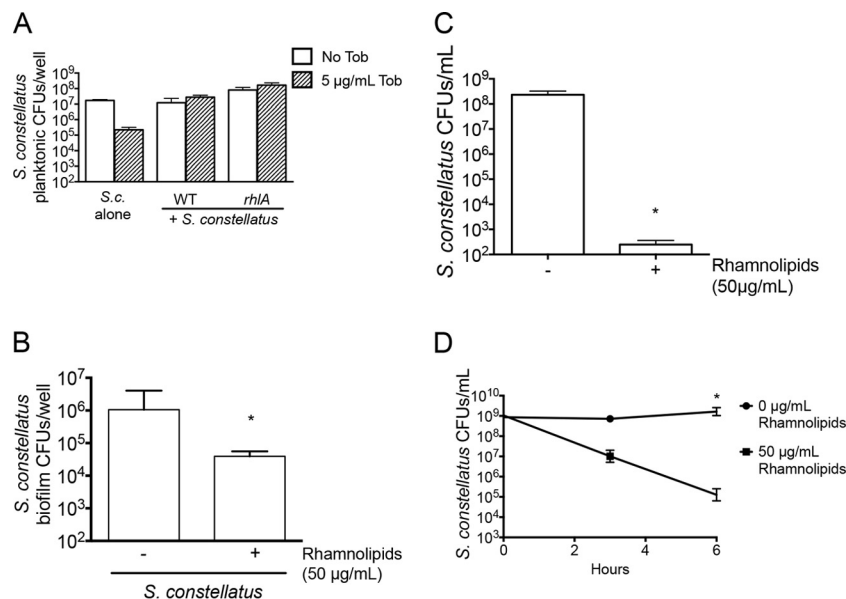


FIG 7 Rhamnolipids directly kill *S. constellatus*. (A) Viability of the planktonic fraction of *S. constellatus* 7155 single and mixed biofilms with the wild type or the *rhIA* mutant of *P. aeruginosa* PA14 on CF airway cells not treated with tobramycin or treated with 5 µg/ml tobramycin. (B) Viability of *S. constellatus* 7155 grown as single biofilms on CF airway cells treated or not treated with 50 µg/ml rhamnolipids, as indicated. *, P value < 0.05 , as determined by Mann-Whitney test. (C) Viability of *S. constellatus* grown planktonically overnight in THY medium treated or not treated with 50 µg/ml rhamnolipids, as indicated. *, P value < 0.05 , as determined by an unpaired *t* test. (D) Viability of planktonic *S. constellatus* 7155 incubated in phosphate-buffered saline (PBS) with or without 50 µg/ml rhamnolipids, as indicated. *, P value < 0.05 , as determined by one-way ANOVA, followed by Tukey's posttest for multiple comparisons. The bars (A to C) and data points (D) indicate the means and the error bars indicate the SD of the results from three biological replicates.

DISCUSSION

Here, we have shown an example of an interspecies interaction between two predominant organisms in the CF airway, *P. aeruginosa* and *S. constellatus*, potentiated by tobramycin, the frontline maintenance therapy antibiotic used to combat pulmonary infections in individuals with CF. We found that physiologically relevant concentrations (41) of *P. aeruginosa*-produced rhamnolipids can directly kill *S. constellatus* biofilms formed on CF airway cells, and our genetic studies suggest that monorhamnolipid production is sufficient for this effect. Furthermore, tobramycin treatment reduced the amount of rhamnolipids produced by *P. aeruginosa*, thus allowing enhanced growth of *S. constellatus* in mixed biofilms with *P. aeruginosa* on plastic and CF airway cells.

It is important to note that the interaction we observed here is specific to a biofilm of *S. constellatus* 7155 and *P. aeruginosa* PA14. We tested other *Streptococcus* strains and other isolates of *P. aeruginosa* but did not observe a similar interaction. At this stage, the basis of this specificity is not understood, but it might result, for example, from the differential production of rhamnolipids by *P. aeruginosa* or from *S. constellatus* 7155 being particularly sensitive to this surfactant. Despite the strain specificity of the infection, this work does illustrate the potential of microbial interactions in the context of host cells to impact antibiotic tolerance in unanticipated ways. It will be of interest if other similar interactions are revealed in the future.

Rhamnolipid production in the lung is varied (44); *P. aeruginosa* strains defective in quorum sensing have been isolated from the lungs of CF patients (45). These isolates would presumably reduce the production of rhamnolipids, thereby diminishing a potentially protective response against *S. constellatus* *in vivo*. However, Duan and colleagues (14) reported that autolysin-2 produced by a clinical isolate of *Streptococcus* from an individual with CF could activate *P. aeruginosa* genes, including those required for rhamnolipid production (14). This result suggests that the presence of *Streptococcus* may increase the production of rhamnolipids *in vivo*, increasing a potentially protective response against *S. constellatus* unless tobramycin is also administered, which we predict would mitigate rhamnolipid-dependent killing. Thus, the regulation of rhamnolipid production in the lung is complex, and further study is warranted to understand the local and global concentrations of rhamnolipids in particular microbial communities in individual patients.

Interactions among *P. aeruginosa* and other members of the CF microbial community have been described *in vitro* and *in vivo* (14, 15, 46–48). *P. aeruginosa* has been shown to utilize *S. aureus* as an iron source, contributing to *S. aureus* lysis in mixed culture (46). Further, *P. aeruginosa* can sense and respond to peptidoglycan shed from Gram-positive bacteria (47). A recent study of volatile metabolites collected from breath samples of individuals with CF suggests that *P. aeruginosa* produces phenazines, small secreted molecules that can serve as alternative electron acceptors (49) in response to 2,3-butanedione (48). The authors hypothesize that 2,3-butanedione is produced by the *S. milleri* group, of which *S. constellatus* is a member, supporting the hypothesis that *P. aeruginosa* and *S. constellatus* may interact *in vivo* in the context of CF.

Riedele and Reichl (15) reported differential effects of the β -lactam antibiotic ceftazidime on broth-grown single and mixed cultures of the CF pathogens *P. aeruginosa*, *B. cepacia*, and *S. aureus*; furthermore, *S. aureus* was protected from ceftazidime treat-

ment in mixed culture. Coupled with the data presented here, the unintended consequence of increasing the viability of a pathogen after antibiotic treatment in mixed culture (with examples using two different classes of antibiotics) underscores the importance of studying the effects of antibiotics in the context of a polymicrobial infection.

In this work, we demonstrate a complex interspecies relationship in a simple two-member community and reveal the molecular mechanism of at least one aspect of the interaction. While the work presented here focuses on one specific interaction, as we better understand how polymicrobial interactions might impact antibiotic tolerance, such findings might impact approaches to clinical care. And while identifying and understanding the network of interactions among CF microbes that contribute to disease with and without antibiotic perturbation are significant challenges, employing emerging CF sputum microbiomic, metagenomic, transcriptomic, and metabolomic studies as a guide to inform hypotheses, we may be able to leverage our expanded model system to discover interspecies interactions and their molecular mechanisms. We suggest that understanding these mechanisms may allow us to better predict how a given sputum community will respond to current antibiotics and may expose opportunities for novel therapeutic interventions. Ultimately, it may be possible to use this model system as a platform to select the best antibiotic(s) for a given microbial community, personalizing treatment plans based on microbial composition in the lungs.

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